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(54) Title: ALPHA AMYLASE FROM THERMOMYCES LANUGINOSUS

(57) Abstract

A recombinant enzyme is described that is useful for preparing bakery products. The enzyme has the following characteristics: (i) α-amylase activity over the range from about 60 °C to about 80 °C; (ii) a molecular weight of about 54-60 kDa when measured by SDS-PAGE, or of about 55265 Da +/- 100 Da when measured by mass spectrometry; (iii) a pI value of about 3.7 (on a Pharmacia IEF gel, 3-9); (iv) a pH optimum of from about 5.8 to about 6; (v) a temperature optimum of from about 60 °C to about 70 °C.

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Alpha amylase from thermomyc s lanuginosus

The present invention relates to an α-amylase enzyme.

The present invention also relates to the use of the α-amylase enzyme in the manufacture of foodstuffs, in particular foodstuff based on starch, and especially a bakery product, such as bread and similar baked products based on wheat flour.

Bread has a limited shelf-life because it physically changes with time. Among other things, the crumb texture becomes firm, this process being generally known as staling. One explanation of the process of staling appears to be that the wheat starch undergoes a process of retrogradation (re-crystallisation) over a period of time, but undoubtedly there are many other factors.

It has been shown that the addition of bacterial α-amylases to bread, and to a lesser extent, fungal and cereal α-amylases, can retard staling by modifying the gelatinised starch so that retrogradation (recrystallisation) is delayed. For example, see Eliasson, A. and Larssen, K. in Cereals In Breadmaking - A Molecular Colloidal Approach, (1993), Marcel Dekker Inc., 351. However, the activity of bacterial amylases is difficult to control during the baking process and in a manner such as to achieve the desired result.

α-Amylases are also traditionally added to the wheat flour used in bread making to improve the baking quality of the flour. Amongst the α-amylases used for this purpose is the fungal α-amylase from *Aspergillus oryzae*, Drapon, R. and Godon, B. in Enzymes And Their Role In Cereal Technology, (1987), Edited by Kinger, J.E. et al, 281-234, and known as the TAKA-amylase.

However, as a means of delaying the staling process in bread, the TAKA-amylase is far too thermolabile. The gelatinisation of starch (i.e. the melting of starch crystals) starts at about 65°C so that by the time gelatinisation begins much of the TAKA amylase added to the wheat flour will have been inactivated. Bacterial α-amylases,

on the other hand, are too thermally stable so that they are not properly inactivated by the baking process and may lead to a gummy product - see Leloup *et al* [(1993) Bioconversion of Cereal Products, pp 79-127, B.Godon Ed.].

An α-amylase has been isolated from *Thermomyces lanuginosus* (*T. lanuginosus*) and was found to have a half-life of 10 minutes at 70°C (Enzyme Microbiol. Technol. (1992), 14, 112-116). This α-amylase was not suggested for use in baking.

EP-A-0579360 and WO 90/11352 disclose α-amylases obtained from *Pyrococcus* and from the bacterium *P. furiosus*, respectively. These α-amylases were not suggested for use in baking.

For optimal baking properties, an α-amylase should have at least two main criteria. First, the enzyme should be active during the starch gelatinisation stage, which occurs from about 60°C to about 70°C. Second, the enzyme should be inactivated during the baking stage, which occurs from about 80°C to about 100°C. Thus, an α-amylase with intermediate heat stability will be suitable for baking purposes.

The present invention seeks to provide an α-amylase that can be beneficial for baking and which can be prepared easily and in large amounts.

According to a first aspect of the present invention there is provided a recombinant enzyme having the following characteristics:

i. α-amylase activity over the range from about 60°C to about 80°C;
ii. a molecular weight of about 54 - 60 kDa when measured by SDS-PAGE, or of about 55265 Da +/- 100 Da when measured by mass spectrometry;
iii. a pI value of about 3.7 (on a Pharmacia IEF gel, 3-9);
iv. a pH optimum of from about 5.8 to about 6;
v. a temperature optimum of from about 60°C to about 70°C.

According to a second aspect of the present invention there is provided a recombinant nucleotide sequence comprising the coding nucleotide sequence shown in SEQ. I.D. No. 1, or a variant, homologue or fragment thereof.

According to a third aspect of the present invention there is provided a recombinant nucleotide sequence comprising the nucleotide sequence shown as SEQ. I.D. No. 1, or a variant, homologue or fragment thereof.

According to a fourth aspect of the present invention there is provided a construct comprising the recombinant nucleotide sequence according to the present invention.

According to a fifth aspect of the present invention there is provided a vector comprising the recombinant nucleotide sequence according to the present invention or the construct according to the present invention.

According to a sixth aspect of the present invention there is provided a cell, tissue or organism comprising the recombinant nucleotide sequence according to the present invention or the construct according to the present invention or the vector according to the present invention.

According to a seventh aspect of the present invention there is provided a transgenic organism comprising the recombinant nucleotide sequence according to the present invention or the construct according to the present invention or the vector according to the present invention or the cell, tissue or organism according to the present invention.

According to an eighth aspect of the present invention there is provided NCIMB deposit No. 40655, NCIMB deposit No. 40656, NCIMB deposit No. 40657 or NCIMB deposit No. 40658.

According to a ninth aspect of the present invention there is provided a method of preparing a bakery product comprising forming a mixture by contacting a wheat-flour dough with an α-amylase and baking the mixture, wherein the α-amylase is a recombinant α-amylase according to the present invention, or is a recombinant α-amylase expressed by the nucleotide sequence of the present invention, which may be present in any of the afore-mentioned nucleotide aspects of the present invention.

According to a tenth aspect of the present invention there is provided a bakery product obtained by use of the recombinant α-amylase according to the present invention, or a recombinant α-amylase expressed by the nucleotide aspect of the present invention.

According to an eleventh aspect of the present invention there is provided the use of a recombinant α-amylase having α-amylase activity over the range from about 60°C to about 80°C in baking.

According to a twelfth aspect of the present invention there is provided a method of preparing a foodstuff comprising forming a mixture by contacting starch or a starch based ingredient with an α-amylase and optionally processing the mixture, wherein the α-amylase is a recombinant α-amylase as defined above or is a recombinant α-amylase expressed by the nucleotide aspect of the present invention.

According to a thirteenth aspect of the present invention there is provided a foodstuff obtained by use of an α-amylase enzyme wherein the enzyme is a recombinant α-amylase as defined above or is a recombinant α-amylase expressed by the nucleotide aspect of the present invention.

According to a fourteenth aspect of the present invention there is provided the use of a recombinant α-amylase having α-amylase activity over the range from about 60°C to about 80°C in preparing a foodstuff.

Other aspects of the present invention include inserting the recombinant α-amylase nucleotide sequence of the present invention into a suitable vector and/or transforming a suitable host organism with the recombinant α-amylase nucleotide sequence of the present invention and/or expressing that recombinant α-amylase nucleotide sequence gene within the host and/or collecting the expressed recombinant α-amylase and/or isolating the recombinant α-amylase.

Further aspects of the present invention include:

i) other than when forming part of the natural genome, a recombinant nucleotide sequence, which may be either an RNA or DNA sequence, that encodes for a recombinant protein according to the first aspect of the present invention, and in particular the DNA sequence shown in SEQ I.D. No. 1;

ii) a nucleotide sequence that is functionally equivalent to the DNA sequence shown in SEQ I.D. No. 1, that is to say a DNA sequence showing substantial (at least 90%) homology (similarity) therewith and coding for a recombinant α-amylase having the same properties of the recombinant α-amylase having the amino acid sequence as set out in SEQ I.D. No. 1, but differing from the DNA sequence of SEQ I.D. No. 1 by virtue only of the degeneracy of the genetic code;

iii) a recombinant nucleotide sequence that is complementary to the DNA sequence shown in SEQ I.D. No. 1, and the equivalent sequence mentioned in ii);

iv) a vector containing the sequences identified in i), ii), and iii) and especially expression vectors containing that sequence downstream from a suitable promoter sequence capable of expressing the recombinant α-amylase when inserted in a suitable host;

v) a transformant when transformed with a vector according to iv), and especially a transformant transformed with an expression vector and capable of expressing the recombinant α-amylase, and especially an *Aspergillus niger* transformant; and

vi) a method for the production of a recombinant α-amylase according to the present invention which comprises culturing a transformant according to v) and recovering from the culture the recombinant α-amylase expressed in the culture by the transformant.

Preferably the enzyme comprises an N-terminal sequence shown as SEQ. I.D. No. 11.

Preferably the enzyme comprises the sequence shown as SEQ. I.D. No. 1 or SEQ. I.D. No. 2, or a variant, homologue or fragment thereof.

Preferably the enzyme is expressed by a nucleotide sequence comprising the coding sequence shown in SEQ. I.D. No. 1, or a variant, homologue or fragment thereof.

Preferably the enzyme is expressed by a nucleotide sequence comprising the nucleotide sequence shown as SEQ. I.D. No.1, or a variant, homologue or fragment thereof.

Preferably the enzyme is obtained or obtainable from *T. lanuginosus*.

Preferably the enzyme is expressed by a member of the *Aspergillus* family.

Preferably the enzyme is expressed by *Aspergillus niger*.

Preferably the enzyme is obtainable from any one of NCIMB deposit No. 40655, NCIMB deposit No. 40656, NCIMB deposit No. 40657 and NCIMB deposit No. 40658.

Preferably, the transgenic organism is a filamentous fungi, preferably an *Aspergillus* fungus, more preferably *Aspergillus niger*.

Preferably, in the method, the recombinant α-amylase is incorporated into the dough in an amount sufficient to provide from 50 to 400 units of α-amylase activity per kg of wheat flour used to form the dough.

Preferably, in the method, the recombinant α-amylase is incorporated into the mixture in an amount sufficient to provide from 50 to 400 units of α-amylase activity per kg of starch or starch based ingredient to form the mixture.

Preferably, in the method, the foodstuff or bakery product is bread.

Preferably, in the use, the recombinant α-amylase is a recombinant α-amylase according to the first aspect of the present invention or is a recombinant α-amylase expressed by the nucleotide aspects of the present invention.

In a first highly preferred embodiment, the recombinant α-amylase enzyme of the present invention has a protein sequence as set out in SEQ.I.D. No. 1 and SEQ.I.D. No. 2.

In a second highly preferred embodiment, the recombinant α-amylase enzyme of the present invention is coded by the nucleotide sequence as set out in SEQ.I.D. No. 1.

In a third highly preferred embodiment, the recombinant nucleotide sequence is the nucleotide sequence as set out in SEQ.I.D. No. 1.

One of the advantages of the recombinant enzyme of the present invention is that when it is used in baking it improves the shelf-life of bread and other products made from a wheat-flour dough. In this regard, it improves the resistance of bread, and other products made from a wheat-flour dough, to staling. In particular, the recombinant α-amylase has a beneficial thermal activity profile for use in baking.

In addition, the recombinant enzyme of the present invention can be beneficially used in combination with other enzymes useful in the preparation of bakery products.

Other advantages are that the recombinant enzyme can be prepared easily, reliably and cheaply and in large amounts. For example, expressing the gene in *Aspergillus niger* has the advantage that purification and recovery procedures of the recombinant α-amylase are simpler. Moreover, expression in *Aspergillus niger* would obviate the need to remove the intron sequences prior to transformation of that host organism with the recombinant nucleotide sequence of the present invention.

The term "recombinant" is used in its normal sense. For example, the term "recombinant enzyme" does not include an enzyme when prepared by expression of the genomic nucleotide sequence coding for the enzyme when that nucleotide sequence is in its natural environment. Thus, the term covers the enzyme when prepared by expression of a recombinant nucleotide sequence coding for the enzyme when that nucleotide sequence is not in the natural environment for the genomic nucleotide coding sequence. Thus the term "recombinant nucleotide" includes recombinant DNA and recombinant RNA. Preferably it means recombinant DNA, and in some instances it preferably means cDNA.

The recombinant enzyme can be used on its own or in combination with one or more other enzymes.

The terms "variant" or "homologue" or "fragment" include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid or amino acid from or to a respective sequence providing the resultant sequence has the respective ability to code for or act as an enzyme according to the present invention, preferably having at least the same activity of the enzyme comprising the sequence shown as the sequence listing SEQ. I.D. No. 1. In particular, the term "homologue" covers homology with respect to similarity of structure and/or similarity of function providing the resultant nucleotide sequence has the ability to code for an enzyme according to the present invention. With respect to sequence homology, preferably there is more than 80% homology, more preferably at least 85% homology, more preferably at least 90% homology, even more preferably at least 95% homology, more preferably at least 98% homology. The

expressions also cover any codon replacement or substitution with another codon coding for the same amino acid or any addition or removal thereof providing the resultant enzyme has α-amylase activity. Thus, the present invention also covers a modified nucleotide sequence in which at least one nucleotide has been deleted, substituted or modified or in which at least one additional nucleotide has been inserted so as to encode an enzyme having the activity of an α-amylase, preferably having an increased α-amylase activity. The above terms are also synonymous with allelic variations of the sequences.

The term "complementary" means that the present invention also covers recombinant nucleotide sequences that can hybridise to the recombinant nucleotide sequences.

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes the nucleotide sequence according to the present invention directly or indirectly attached to the promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the *Sh1*-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. The terms do not cover the natural combination of the wild type α-amylase gene ordinarily associated with the wild type gene promoter and the wild type promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a fungus or a plant cell into which it has been transferred. Various markers exist which may be used in, for example, plants - such as mannose. Other examples of markers include those that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

The construct of the present invention preferably comprises a promoter.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression. Examples of suitable promoters are those that can direct efficient expression of the nucleotide sequence of the present invention and/or in a specific type of cell. The promoter could additionally include conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable examples of such sequences include the *Sh1*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' leader sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

The terms "obtained" and "obtainable" from *T. lanuginosus* include products obtained directly from cultures of transformed *T. lanuginosus* and products obtained by expression of a recombinant α-amylase gene of the present invention obtainable from *T. lanuginosus* when the gene is in another cellular organism. Preferably, the terms cover expression of the recombinant α-amylase gene of the present invention in another cellular organism, preferably *Aspergillus niger*.

The term "vector" includes an expression vector and a transformation vector. The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression. The term "transformation vector" means a construct capable of being transferred from one species to another - such as from an *E.Coli* plasmid to a fungus or a plant cell, or from an *Agrobacterium* to a plant cell.

The terms "cell", "tissue" and "organ" include cell, tissue and organ *per se* and when within an organism.

The term "organism" in relation to the present invention includes any organism that could comprise the recombinant nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the recombinant nucleotide sequence according to the present invention can be expressed when present in the organism. Preferably the organism is an α-amylase producing organism such as any one of a plant, algae, fungi, yeast and bacteria, as well as cell lines thereof. Preferably the organism is a filamentous fungus, preferably of the genus *Aspergillus*, more preferably *Aspergillus niger*.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the recombinant nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the recombinant nucleotide sequence according to the present invention can be expressed within the organism. Preferably the recombinant nucleotide sequence is incorporated in the genome of the organism. Preferably the transgenic organism is a filamentous fungus, preferably of the genus *Aspergillus*, more preferably *Aspergillus niger*.

If the organism can not naturally splice intron regions post expression then a pre-transformation step may be necessary wherein any intron sections are removed.

The host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Sambrook *et al*. in Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the recombinant nucleotide sequence of the present invention may need to be suitably modified before transformation - such as by removal of introns.

As mentioned above, a preferred host organism is of the genus *Aspergillus*, such as *Aspergillus niger*.

The transgenic *Aspergillus* according to the present invention can be prepared by following the teachings of Rambosek, J. and Leach, J. 1987 (Recombinant DNA in filamentous fungi: Progress and Prospects. CRC Crit. Rev. Biotechnol. 6:357-393), Davis R.W. 1994 (Heterologous gene expression and protein secretion in *Aspergillus*. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp 525-560), Ballance, D.J. 1991 (Transformation systems for Filamentous Fungi and an Overview of Fungal Gene structure. In: Leong,S.A., Berka R.M. (Editors) Molecular Industrial Mycology. Systems and Applications for Filamentous Fungi. Marcel Dekker Inc. New York 1991. pp 1-29) and Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666). However, the following commentary provides a summary of those teachings for producing transgenic *Aspergillus* according to the present invention.

Filamentous fungi have been widely used in industry for production of organic compounds and enzymes. Traditional japanese koji and soy fermentations have used *Aspergillus sp* for hundreds of years. In this century *Aspergillus niger* has been used for production of organic acids particular citric acid and for production of various enzymes for use in industry.

There are two major reasons for that filamentous fungi have been so widely used in industry. First filamentous fungi can produce high amounts of extracellular products, for example enzymes and organic compounds such as antibiotics or organic acids. Second filamentous fungi can grow on low cost substrates such as grains, bran, beet pulp etc. The same reasons have made filamentous fungi attractive organisms as hosts for heterologous gene expression according to the present invention.

In order to prepare the transgenic *Aspergillus*, expression constructs are prepared by inserting the recombinant nucleotide sequence of the present invention into a construct designed for expression in filamentous fungi. In this regard, several types of constructs used for heterologous gene expression have been developed. The

constructs will contain a promoter which is active in fungi. Examples of promoters include a fungal promoter for a highly expressed extracellular enzyme, such as an glucoamylase promoter or an α-amylase promoter. The recombinant nucleotide sequence of the present invention can be fused to a signal sequence which directs the enzyme encoded by the nucleotide sequence to be secreted. Usually a signal sequence of fungal origin is used. A terminator active in fungi ends the expression system.

Another type of expression system has been developed in fungi where the recombinant nucleotide sequence of the present invention is fused to a smaller or a larger part of a fungal gene encoding a stable protein. This can stabilize the protein encoded by the recombinant nucleotide sequence of the present invention. In such a system a cleavage site, recognized by a specific protease, can be introduced between the fungal protein and the enzyme encoded by the recombinant nucleotide sequence of the present invention, so the produced fusion protein can be cleaved at this position by the specific protease thus liberating the protein or enzyme encoded by the recombinant nucleotide sequence of the present invention. By way of example, one can introduce a site which is recognized by a KEX-2 like peptidase found in at least some *Aspergilli*. Such a fusion leads to cleavage *in vivo* resulting in protection of the recombinant enzyme and production of the recombinant enzyme and not a larger fusion protein or enzyme.

Heterologous expression in *Aspergillus* has been reported for several genes coding for bacterial, fungal, vertebrate and plant proteins or enzymes. The proteins can be deposited intracellularly if the recombinant nucleotide sequence of the present invention is not fused to a signal sequence. Such proteins or enzymes will accumulate in the cytoplasm and will usually not be glycosylated which can be an advantage for some bacterial proteins or enzymes. If the recombinant nucleotide sequence of the present invention is equipped with a signal sequence the enzyme will accumulate extracellulary.

With regard to product stability and host strain modifications, some heterologous proteins or enzymes are not very stable when they are secreted into the culture fluid of fungi. Most fungi produce several extracellular proteases which degrade heterologous proteins or enzymes. To avoid this problem special fungal strains with reduced protease production have been used as hosts for heterologous production.

For the transformation of filamentous fungi, several transformation protocols have been developed for many filamentous fungi. Many of them are based on preparation of protoplasts and introduction of DNA into the protoplasts using PEG and Ca^{2+} ions. The transformed protoplasts then regenerate and the transformed fungi are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as *argB*, *trpC*, *niaD* and *pyrG*, antibiotic resistance markers such as benomyl resistance, hygromycin resistance and phleomycin resistance. A very common used transformation marker is the *amdS* gene of *A. nidulans* which in high copy number allows the fungus to grow with acrylamide as the sole nitrogen source.

In another embodiment the transgenic organism can be a yeast. In this regard, yeast have been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by King *et al* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability.

Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

An additional advantage is that yeasts are capable of post-translational modifications of proteins and thereby have the potential for glycosylation and/or secretion of heterologous gene products into the growth medium.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Also, yeasts are known to secrete very few proteins into the culture medium. This makes yeast a very attractive host for expression of heterologous genes, since secretable gene products can easily be recovered and purified.

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the recombinant nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the recombinant nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. The recombinant nucleotide sequence of the present invention can be fused to a signal sequence which directs the enzyme encoded by the nucleotide sequence to be secreted. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For expression in yeast, it may be advantageous to remove some or all of the intron or intron sequences.

Heterologous expression in yeast has been reported for several genes. The gene products can be glycosylated which is advantageous for some enzymes intended for specific application where heat tolerance is desirable. The protein or enzyme can be deposited intracellularly if the recombinant nucleotide sequence of the present invention is not fused to a signal sequence, or they can be secreted extracelluarly if the recombinant nucleotide sequence of the present invention is equipped with a signal sequence.

For the transformation of yeast several transformation protocols have been developed. The transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen *et al* (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

The present invention relates to the manufacture of a foodstuff. The foodstuff may be based on starch and can be, for example, any one of bread, pasta, noodles etc.

However, in a preferred aspect, the present invention provides a method of improving the shelf-life and resistance to staling of bread and other products made from wheat flour dough which comprises incorporating into the dough an α-amylase according to the first aspect of the present invention. Typically, the recombinant α-amylase is incorporated into the dough in an amount sufficient to provide from 50 to 400 units of α-amylase activity per kg of wheat flour used to form the dough.

The recombinant α-amylase can be added to the dough at any convenient time during the mixing process, and may be used in conjunction with other enzymes, such as enzymes added as dough improvers, which may also be added in the conventional manner.

Thus, the recombinant α-amylase enzyme of the present invention may be added in combination with other enzymes or chemical additives, such as emulsifiers, rheology modifiers, stabilisers, surfactants, preservatives, flavourings, colourings etc.

Typically, the other ingredients and procedures used to form the dough and to bake the dough will be conventional and can be found in any relevant textbook.

A number of plasmids have been prepared in accordance with the present invention by adaptation, including *in vivo* excision, of ZAPII phage. These plasmids, which have been called pAMYA-1, pAMYA-2, pAMYA-3 and pAMYA-5, contain the α-amylase gene of the present invention. pAMYA-1 is 8.48 Kb in size, pAMYA-2 is 7.98 Kb in size, pAMYA-3 is 9.98 Kb in size and pAMYA-5 is 10.48 Kb in size.

E. coli containing these plasmids have been deposited with the National Collections of Industrial and Marine Bacteria (NCIMB), 23 St. Machar Drive, Aberdeen AB2 1RY, United Kingdom, on 23 June 1994 under the deposit numbers:

pAMYA-1: NCIMB 40655
pAMYA-2: NCIMB 40656
pAMYA-3: NCIMB 40657
pAMYA-5: NCIMB 40658

A further plasmid has been prepared in accordance with the present invention. This plasmid has been called pPR28. This plasmid also contains the α-amylase gene of the present invention. This plasmid has 6573 base pairs.

The present invention will now be described only by way of examples, in which reference shall be made to the following Figures:

Figure 1 is a restriction map of plasmid pAMYA-1;

Figure 2 is a restriction map of plasmid pAMYA-2;

Figure 3 is a restriction map of plasmid pAMYA-3;

Figure 4 is a restriction map of plasmid pAMYA-5;

Figure 5 is a restriction map of plasmid pPR28;

Figure 6 is a graph used to determine a pH optimum;

Figure 7 is a graph used to determine a temperature optimum;

Figure 8 contains plots of a starch degradation study; and

Figure 9 contains plots of a baking study.

In the following experiments, α-amylase activity is determined by use of the following protocol.

Protocol For Measurement of α-Amylase Activity

α-amylase activity was analysed by determining the degree of hydrolysis of a standard starch solution (modification of method described by Sandstedt et al. 1939). The reaction mixture consisted of 2.5 ml of a 2% (w/v) solution of soluble wheat starch in 0.1 M sodium acetate buffer, 20 mM $CaCl_2$ (pH 4.8) and 0.5 ml of an enzyme solution. After 12, 14, 16, 18 and 20 minutes 0.5 ml extracts of the reaction mixture were transferred to 2.5 mls of a I_2-KI solution (0.7 mM I_2 and 241 mM KI).

The absorbance of the blue-coloured complex between starch and I_2-KI was then measured at 620nm against water. The time needed to reach an absorbance of 0.48 was determined and used to calculate enzyme activity:

$$\text{Activity} = \frac{0.1 \times 60 \times 1000 \text{ FAU/g}}{A \times T}$$

A = enzyme dilution (mg enzyme/ml solution)

T = time (min) necessary to reach an absorbance of 0.48.

1 Fungal Amylase Unit (FAU) is the quantity of enzyme which will dextrinise the standard starch solution at a rate of 1 g per hour at 40°C.

During purification, amylase activity was estimated by adding 25-100µl of the enzyme solution to 1 ml of the standard starch solution at 50°C and transferring 0.5 ml of the reaction mixture to 2.5 ml I_2 -KI solution after 5-10 minutes. Absorbance at 620 nm of the starch-iodine solution was used as an arbitrary activity unit.

1. Construction of *T. lanuginosus* genomic library

1.1. Source Organism

The fungus *Thermomyces lanuginosus* was used as the source organism to generate DNA for a genomic library for subsequent studies and procedures. The *Thermomyces lanuginosus* used was strain CBS 224.63 obtained from Centraalbureau voor Schimmelcultures, Delft (CBS). A sample of this strain was grown on YPS agar plates at 40°C (yeast extract 4 g/l, wheat starch 15 g/l, K_2HPO_4 1 g/l, $MgSO_4$ 0.5 g/l and agar 20 g/l).

1.2. DNA extraction

1g of frozen *T. lanuginosus* mycelium was ground in a mortar under liquid nitrogen. Following evaporation of the nitrogen cover, the ground mycelium was extracted with 15ml of an extraction buffer (100mM Tris·HCl,pH8.0, O.50mM EDTA, 500mM NaCl, 10mM ß-mercaptoethanol) containing 1ml 20% sodium dodecyl sulphate. After incubation at 65°C for 10 min. 5ml 5M KAc. pH 5.0, was added and the mixture further incubated, after mixing, on ice for 20 mins. The mixture was then centrifuged for 20 mins. and the supernatant mixed with 0.6 vol. isopropanol to precipitate the extracted DNA. After further centrifuging for 15 mins. the DNA pellet was dissolved in 0.7 ml TE (10mM Tris, HCl pH 8.0, 1mM EDTA) and precipitated with 75 µl 3M NaAc, pH 4.8, and 500 µl isopropanol.

After centrifuging the pellet was washed with 70% ETOH and dried under vacuum. The DNA was dissolved in 200 µl TE and stored at -20°C.

1.3 DNA digestion and cloning

40 µg of the DNA prepared as in 1.2 was digested with 1 U Sau3A in a 500 µl volume. 125 µl digests were removed after 5, 10, 15 and 20 minutes. Using the 10 and 15 minute digests, the digested DNA was precipitated, dissolved in 20 µl water, mixed with a 50-fold excess of a double stranded DNA adapter having the sequences shown as SEQ I.D. No. 3 and SEQ I.D. No. 4 which has Sau3A and EcoRI overhangs at either end and two internal restriction sites, and ligated with T4 ligase overnight.

The adaptor can be prepared by annealing equal amounts of the nucleotide sequence shown as SEQ. I.D. No. 3 phosphorylated with T4 polynucleotide kinase and the nucleotide sequence shown as SEQ. I.D. No. 4.

After ligation, T4 ligase activity was destroyed by heating for 15 min. at 65°C. The fragments were then phosphorylated with T4 polynucleotide kinase.

The two separate DNA preparations were run on a 1.0% preparative agarose gel and the DNA fragments in the size range 5-10kb were recovered and combined.

150 ng of the combined 5-10kb fragments were then mixed with 1μg of an EcoRI predigested and dephosphorylated λZAPII vector (Stratagene #236211) and ligated. The ligated vectors were packed in vitro with Gigapack II Gold packing extracts (Stratagene #200214), and the titre determined.

The resultant library contained 300,000 independent phages of which about 95% contained inserts.

2. Construction of a *T. lanuginosus* DNA probe.

2.1 Construction of PCR Primers.

By comparisons of the known and published protein sequences of the α-amylase genes from *Schwanniomyces occidentalis*, *Saccharomycopsis fibuligera* and *Aspergillus oryzae* (Swiss protein release 24 database) two conserved regions of the α-amylase gene were identified, being respectively the sequences:

Met-Glu-Phe-Thr-Ala-Ile-Trp-Ile
M G P T A I W I

and

Tyr-Thr-Cys-Pro-Tyr-Gln-Asn
Y T C P Y Q N

Corresponding oligonucleotides were then synthesised for each of the possible DNA sequences encoding for those two sequences, taking the degeneracy of the genetic code into account, and for use as degenerate primers in the subsequent PCR amplification of genetic DNA from *T. lanuginosus*. Collectively those degenerate primers are represented by the sequences SEQ I.D. No. 5 (Primer 1) and SEQ I.D. No. 6 (Primer 2).

2.2 PCR Cloning of the *T. lanuginosus* α-amylase gene

1 μg of *T. lanuginosus* DNA in a 100 μl volume was amplified by PCR using 100 pmol of each of the Primers 1 and 2 according to the following programme:

STEP 1	97°C	10 min.
STEP 2	60°C	10 min., polymerase added
STEP 3	94°C	1 min
STEP 4	50°C	2 min
STEP 5	72°C	2 min
STEP 6	5°C	indefinite

STEPS 3-5 were repeated for 40 cycles.

The reaction produced a ca. 800nt fragment which was cloned in pT7-Blue (Novagen #69829-1). Both ends of the fragment were sequenced using a commercial sequencing kit (USB: Sequenase) using a universal primer and a T7 primer.

The determined DNA sequences and the deduced amino acid sequences are set out herein as SEQ I.D. Nos. 7 and 8, and SEQ I.D. Nos. 9 and 10.

3. Isolation of *T. lanuginosus* α-amylase gene

3.1 2 x 30,000 independent clones of the *T. lanuginosus* gene library were plated on 22 x 22 NZY plates (per liter 5g NaCl, 2g $MgSO_4.7H_2O$, 5g Yeast extract, 10g casein hydrolysate, 20g agar, pH 7.5) and plaques obtained. Two replicas of each plate were made on Hybond N membranes (Amersham) and treated according to manufacturer's instructions.

3.2 The *T. lanuginosus* α-amylase gene fragment obtained as in 2.1 above was labelled with α-^{32}P-dCTP using a "Prime-It" random primer labelling kit (Stratagene #300385) and used to probe the membranes obtained as in 3.1. After 24 hours

hybridisation period, the membranes were washed according to the manufacturer's instructions, autoradiographed, and the positive plaques isolated. For this purpose, a positive plaque was declared only if it hybridised to the probe on both membranes.

Plaques containing the full length gene were identified. Phages were extracted from the isolated plaques and purified by plating on small NYZ plates and repeating the plaque lift and hybridisations. The purified clones were sub-cloned *in vivo* by the ExAssist system (Stratagene #200253). The resulting plasmids with the full length α-amylase gene have been named pAMYA 1-3 and 5, and their restriction maps are shown in Figures 1 - 4.

4. Sequencing of the *T. lanuginosus* α-amylase gene

4.1 The *T. lanuginosus* gene was sequenced using an ALF (Pharmacia) sequencer. The complete sequence, which is shown as SEQ I.D. No. 1, comprises eight introns and nine exons.

5. Expression of recombinant α-amylase

5.1 **Transformation**

An *Aspergillus niger* strain, 3M43 (Danisco Ingredients strain collection) was transformed with a construct containing the promoter and the terminator of the xylanase-A gene from *A. niger* (*xlnA*) and the structural gene for amylase, *amyA*, from *T. lanuginosus*. The resultant plasmid is pPR28 is shown in Figure 5.

The *xylA* gene has been described by Graaff et al. (de Graaff, L.H. et al. (1994) Molecular Microbiology 12(3), 479-490). The xylanase-promoter is a 427 bp BspHI fragment ending immediately before the ATG start codon of *xlnA*. A 1079 bp BamHI-XbaI fragment contains the *xlnA* terminator. This fragment starts 64 bp downstream of the translation stop codon of the *xlnA* gene.

A 2106 bp, ScaI-Ecl136II fragment of the *amyA* gene contains the protein coding region. This fragment starts 21 bp before the *amyA* start codon and ends 68 bp after the stop codon.

The fragments were assembled in pBluescript II SK+ (Stratagene) which was transformed into *A. niger* using a co-transformation procedure (Werners, K. et al. (1987), Molecular and General Genetics 209, 71-77).

5.2 Purification of the transgenic α-amylase

The transformants were grown in shake flasks (250 ml with 100 ml substrate) in 100 mM MES, pH 5.5 with 2% wheat bran, 2% beet pulp and 0.2% KNO_3. After 6 days' growth a high α-amylase activity was found in the cultures of the transformants whereas the untransformed control did not produce amylase.

The amylolytic activity was purified as follows: 20 ml of filtered culture material was desalted on PD10 columns (Pharmacia) equilibrated with 20 mM triethanolamine, pH 7.3. 30 ml of the desalted material was applied to an anion exchange column, Q-Sepharose Fast Flow (21 x 2.5 cm, flow 1.5 ml/min, Pharmacia) equilibrated with 20 mM triethanolamine, pH 7.3. A NaCl gradient was applied by adding triethanolamine, pH 7.3 with 1 M NaCl (buffer B) as follows: 0 - 25% buffer B in 300 min. and thereafter 25 - 50% buffer B in 700 min. Fractions of 7.5 ml were - collected.

The α-amylase containing fractions were pooled (total volume 72 ml) and concentrated to a volume of 10 ml. This sample was loaded onto a gel filtration column, Superdex G75 Hiload (60 x 2.5 cm) equilibrated with 50 mM sodium acetate buffer containing 0.1 M NaCl, pH 5.8. Protein was eluted with a flow of 1.66 ml/min and fractions of 3.3 ml were collected. Samples of α-amylase containing fractions were analysed by SDS-PAGE on a 4-12% Tris-glycine gel. The α-amylase containing fractions gave a broad band at 54 - 60 kDa. The α-amylase containing fractions were pooled and frozen for further analysis.

5.3 Characterization of the recombinant α-amylase

5.3.1 N-terminal Sequence

The N-terminal of the purified α-amylase was determined according to conventional procedures (Matsudaira, P.: A Practical Guide to Protein and Peptide Purification for Microsequencing. Second Edition. Academic Press, San Diego 1993) and was found to have the following sequence:

A T P D E W K A Q S

The sequence, which is identical to the amino acid sequence (amino acids number 1 to 10) in SEQ I.D. No. 1, is shown as SEQ I.D. No. 11. This confirms that the purified α-amylase is derived from the introduced *Thermomyces lanuginosus* α-amylase gene.

5.3.2 Molecular weight

As mentioned above, the MW of the purified α-amylase on a Tris-glycine gel was determined to be 54 - 60 kDa. The MW was 55265 +/- 100 Da when determined by mass spectrometry. Specific colouring of the gel showed that the α-amylase is a glycoprotein which was also confirmed by mass spectrometry.

The MW is 51.737kDa when calculated from the amino acid sequence and the enzyme probably has two N-glycosylated sites.

5.3.3 pI

pI of the α-amylase was determined by use of a Pharmacia IEF gel, 3-9, and was measured to be approximately 3.7.

5.4 Activity measurements

Activity measurements for determination of pH and temperature optimum were made by following the formation of reducing equivalents in a 0.08% starch solution (described in detail below). The reaction was stopped by adding an alkaline sodium phosphate solution (0.112 M Na_2HPO_4, 2 H_2O; 55 mM NaOH in distilled water). Then a colour reagent (3.5 mM $K_3(Fe(CN)_6$; 56 mM Na_2HPO_4, 2 H_2O; 55 mM NaOH in distilled water) was added. The samples were boiled for 5 min. After cooling, 300 µl of the samples were transferred to a microtiter plate and absorbance at 405 nm was measured. Activity was determined from absorbance differences between samples and a blank. Glucose was used as a standard.

The enzyme solution contained an enzyme activity of 176 µmol reducing equivalents/min/ml when measured in an acetate buffer, pH 6 (acetic acid) at 50°C.

5.4.1 pH optimum

pH optimum was determined as follows: To 400 µl 0.1% starch solution in 100 mM of either MES (pH 4.8 to 7) or MOPS (pH 7 to 8) buffers was added 75 µl of enzyme solution (0.16 µmol reducing equivalents/min/ml). After 6 min. the activity was stopped by adding 240 µl stop solution. Then 360 µl colour reagent was added, and the samples were boiled for 5 min. Activity was determined as described above.

The pH optimum of the recombinant α-amylase of the present invention was measured to be from 5.8 to 6 (see Figure 6).

5.4.2 Temperature optimum

Temperature optimum was determined as follows: 75 µl 0.5% starch solution in 100 mM sodium acetate buffer, pH 6 (acetic acid) was placed in a water bath thermostatted at the measuring temperature. Then 400 µl α-amylase (29 nmol reducing equivalents/min/ml) was added. Enzyme activity was stopped after 5 and 8

min., respectively by adding 240 µl stop solution. 360 µl colour reagent was added and activity was determined as described above.

Results are shown in Figure 7. The same results were obtained when 100 mM MES buffer at pH 6 was used. The temperature optimum of the recombinant α-amylase of the present invention was determined to be between 60 and 70°C.

5.5 Heat stability

Heat stability was determined as follows: 600 µl 100 mM sodium acetate buffer, pH 6 (acetic acid) was placed in a water bath maintained at the measuring temperature. 50 µl enzyme solution was added and after appropriate time intervals 100 µl samples were taken out and immediately added to 750 µl acetate buffer which was kept on ice.

α-Amylase activity in these samples was determined by incubating 400 µl of the samples with 75 µl 0.5% starch solution in 0.1 M acetate buffer, pH 6 at 50°C for 10 min. 240 µl stop solution and 360 µl colour reagent were added and activity was determined as described above.

Heat inactivation of the enzyme is an exponential function of time and heat stability at different temperatures can be described by the half times of inactivation. The results are presented in Table 1.

TABLE 1

temperature (°C)	half time (min)
60	89
65	29
70	16
75	6
80	3
85	2

5.6 Starch degrading profile

To 2.5 ml 5% starch solution in water was added 250 µl enzyme solution (1.5 µmol reducing equivalents/min/ml). The samples were incubated at 50°C and enzyme activity was stopped by boiling the samples after 0.5, 1, 1.5, 2, 3, 4 and 5 hours, respectively. The samples were diluted 20 times in water and analysed on a Dionex HPAEC system on a CarboPac Pa-1 column (4 x 250 mm, flow 1 ml/min) with 0.2 M NaOH, 1.5 M NaOAc and water as eluents in a gradient optimized for separation of glucose oligomers. 0.3 M NaOH was added after the separation column and the dextrins were detected by PAD (pulse amperometric detection). 20 µl samples were applied to the column.

The results are shown in Figure 8, which show the oligomer pattern in starch samples when treated with α-amylase as mentioned above. B0 is the control (i.e. no enzyme, stopped after 5 hrs), whereas the other plots are for the enzyme of the present invention wherein B1 is stoppage at 0.5 hrs, B2 is stoppage at 1 hrs, B3 is stoppage at 1.5 hrs, B4 i stoppage at 2 hrs, B5 is stoppage at 3 hrs, B6 is stoppage at 4 hrs and B7 is stoppage at 5 hrs. In Figure 8, the peak identification is that for glucose, retention time 7.1 min (in B7, glucose peaks at retention time 11.2 which can not be explained); maltose, retention time 15.9 min.; maltotriose, retention time 17 min. and maltotetraose, retention time 18 min.

The results show that the recombinant α-amylase of the present invention is endo-specific, producing only a little glucose. After 5 hrs., only 4% of the total peak area is due to glucose, whereas maltose accounts for 39%.

6. Baking Study

In this example, the beneficial effect of the α-amylase of the present invention in the manufacture of bread is illustrated.

Bread was prepared as follows:

Recipe:	flour	2000 g
	dry yeast	30 g
	sugar	30 g
	salt	30 g
	water	400 BU+ 60 g

BU refers to Brabender units

Procedure:	kneading	Hobart
		2 + 12 min.
		(depending on flour)
	dough temperature	24°C
	resting	10 min. at 30°C
	scaling	2 x 375 g
	resting	5 min. at 33°C, 85% RH
	moulding	mono 11
	proofing	50 min. at 33°C, 85% RH
	baking	40 min. at 220°C
		10 sec. steam

Enzyme addition:

1. control, no addition
2. TAKA amylase, 100 FAU/kg flour
3. Recombinant α-amylase of the present invention, 100 FAU/kg flour
4. TAKA amylase, 350 FAU/kg flour
5. Recombinant α-amylase of the present invention, 350 FAU/kg flour

Handling properties and stickiness of the dough were evaluated during preparation of the bread and after baking the crumb was evaluated.

After baking, the bread was stored in plastic bags at 20°C. Firmness of the bread crumb was measured on day 3 and 7 using an Instron apparatus. 8 slices from each bread loaf were measured and the mean value was calculated. The results are shown in Figure 9.

As can be seen from Figure 9, there is a dosage effect of the recombinant α-amylase of the present invention. Furthermore, the enzyme of the present invention clearly has a beneficial effect on firmness.

Furthermore, the recombinant α-amylase of the present invention lowers firmness as compared to a high dosage of TAKA amylase, meaning that it is a better anti-staling enzyme than the TAKA amylase.

Dough handling properties were similar for doughs prepared with TAKA amylase and with the recombinant α-amylase of the present invention.

The crumb of bread with the recombinant α-amylase of the present invention was evaluated and was found to be more moist than the crumb of bread baked with TAKA amylase.

In summation the present invention relates to a recombinant α-amylase and its use in baking. Other aspects of the present invention include the deposited microorganisms, and mutants, variants and progeny obtained directly or indirectly therefrom and retaining the ability, when cultured, of producing a recombinant α-amylase having the activity described herein.

Other modifications of the present invention will be apparent to those skilled in the art.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: DANISCO A/S
(B) STREET: Langebrogade 1, P.O. Box 17
(C) CITY: Copenhagen
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): 1001
(G) TELEPHONE: +45 32 66 22 00
(H) TELEFAX: +45 32 66 21 67

(ii) TITLE OF INVENTION: Alpha amylase from Thermomyces lanuginosus

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3311 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Thermomyces lanuginosus

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:join(427..585, 660..698, 755..876, 956..1064,
1142..1370, 1425..1584, 1637..1783, 1846..2086,
2168..2443)

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION:427..480

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:join(481..585, 660..698, 755..876, 956..1064,
1142..1370, 1425..1584, 1637..1783, 1846..2086,
2168..2440)

(ix) FEATURE:
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(B) LOCATION:427..585

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:586..659

(ix) FEATURE:
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(B) LOCATION:660..698

(ix) FEATURE:
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(B) LOCATION:699..754

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(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION:2087..2167

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:2168..2443

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CTTTGTCCAA ACATTTGCCT AATTGCCCAT CACCAACGGC TTTAAGAATC ATGTACCTCG     120

CTCATTTGAG AAGATGGAAG ATGCCATTGC CACGGCAGTC GATCAATCTA CTGGCTATGG     180

ATGGTCCGAA TTTTCCGTCG TCGCCTTTTG CTCTAATCTC GGTATGACCT TCACCGAGCG     240

AATCGTAGAA TATTTAAAGG GTTGATCGAG CCACTTTGTC GCCGATGTCT GACTCTGTCG     300

TGTCACAACT GAACTGATCA GACGAGGTCA TCTGAGTCTC TCGTCGAAAA AAGACTCGTT     360

GGATGATTCT CGATTTCAAC TCGGGACAAG TCATAGAACG AGTAGTACTT CAGTTGGCCA     420

CAAAAG ATG AAG TCT CTC GCC GCA ATT GCT GCT CTG CTG TCG CCC ACA          468
       Met Lys Ser Leu Ala Ala Ile Ala Ala Leu Leu Ser Pro Thr
       -18         -15                 -10                 -5

CTG GTC CGG GCA GCG ACT CCG GAT GAG TGG AAA GCT CAG TCG ATC TAT       516
Leu Val Arg Ala Ala Thr Pro Asp Glu Trp Lys Ala Gln Ser Ile Tyr
                 1               5                  10

TTC ATG CTG ACG GAC CGG TTT GCG CGT ACC GAC AAT TCG ACC ACG GCT       564
Phe Met Leu Thr Asp Arg Phe Ala Arg Thr Asp Asn Ser Thr Thr Ala
        15                  20                  25

CCC TGT GAC ACC ACT GCC GGG GTATGCAACT AACCCTGTGT TTCTCTTCCC          615
Pro Cys Asp Thr Thr Ala Gly
     30                  35

GGGACGTACA AGGGGTCTTC TCCATGCTAA CCGTGCACAT GCAG AAA TAT TGC GGG      671
                                                 Lys Tyr Cys Gly

GGA ACA TGG CGA GGT ATC ATC AAC AAC GTAAGTGGCT TCTGATTTTC             718
Gly Thr Trp Arg Gly Ile Ile Asn Asn
 40                  45

GCTCAATAAT CTTCGTCGCG TGACTTTATT TCCTAG CTG GAT TAC ATC CAG GAT       772
                                       Leu Asp Tyr Ile Gln Asp
                                            50

ATG GGC TTC ACA GCT ATC TGG ATA ACT CCA GTG ACA GCC CAG TGG GAC       820
Met Gly Phe Thr Ala Ile Trp Ile Thr Pro Val Thr Ala Gln Trp Asp
 55                  60                  65                  70

GAC GAT GTG GAT GCG GCA GAT GCA ACG TCG TAT CAC GGT TAT TGG CAG       868
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Asp Asp Val Asp Ala Ala Asp Ala Thr Ser Tyr His Gly Tyr Trp Gln
                  75                  80                  85

AAA GAC CT  GTGCGCAACC CTGCTCCATG GATCGCTGGC TGCAAACTCG                916
Lys Asp Leu

TGCTGATCGG TGATTTTTTT TTTTTTTTTT TTGAAACAG A TAC TCT CTG AAT TCG       971
                                          Tyr Ser Leu Asn Ser
                                           90

AAA TTC GGC ACT GCC GAT GAC TTG AAA GCC CTG GCT GAT GCC CTT CAC       1019
Lys Phe Gly Thr Ala Asp Asp Leu Lys Ala Leu Ala Asp Ala Leu His
 95                 100                 105                 110

GCC CGT GGG ATG CTT CTC ATG GTC GAC GTC GTG GCT AAT CAC TTT           1064
Ala Arg Gly Met Leu Leu Met Val Asp Val Val Ala Asn His Phe
               115                 120                 125

GTACGGACCA TCTACATACC TGGGAAACGC GAAGAAGGAA AAAAAAAAAA AGGCGCACGC     1124

TAACATTTCG CGTTTAG GGC TAC GGC GGT TCT CAT AGC GAG GTG GAT TAC        1174
                    Gly Tyr Gly Gly Ser His Ser Glu Val Asp Tyr
                                  130                 135

TCG ATC TTC AAT CCT CTG AAC AGC CAG GAT TAC TTC CAC CCG TTC TGT       1222
Ser Ile Phe Asn Pro Leu Asn Ser Gln Asp Tyr Phe His Pro Phe Cys
           140                 145                 150

CTC ATT GAG GAC TAC GAC AAC CAG GAA GAA GTC GAA CAA TGC TGG CTG       1270
Leu Ile Glu Asp Tyr Asp Asn Gln Glu Glu Val Glu Gln Cys Trp Leu
       155                 160                 165

GCC GAT ACT CCG ACG ACA TTG CCC GAC GTG GAC ACC ACC AAT CCT CAG       1318
Ala Asp Thr Pro Thr Thr Leu Pro Asp Val Asp Thr Thr Asn Pro Gln
    170                 175                 180

GTT CGG ACG TTT TTC AAC GAC TGG ATC AAG AGC CTG GTG GCG AAC TAC       1366
Val Arg Thr Phe Phe Asn Asp Trp Ile Lys Ser Leu Val Ala Asn Tyr
185                 190                 195                 200

TCC A GTATGATTGT TCCCGCGGTA ACGCTTTAGG GCTTGCTCTA ACTGAAATCG          1420
Ser

ACAG  TC GAT GGT CTG CGC GTC GAC ACC GTT AAG CAC GTG GAG AAA GAT      1468
     Ile Asp Gly Leu Arg Val Asp Thr Val Lys His Val Glu Lys Asp
                  205                 210                 215

TTC TGG CCC GAC TTC AAC GAA GCT GCT GCG TGT ACC GTC GGC GAG GTG       1516
Phe Trp Pro Asp Phe Asn Glu Ala Ala Ala Cys Thr Val Gly Glu Val
           220                 225                 230

TTC AAC GGT GAC CCA GCG TAC ACC TGC CCA TAC CAG GAA GTG CTG GAT       1564
Phe Asn Gly Asp Pro Ala Tyr Thr Cys Pro Tyr Gln Glu Val Leu Asp
       235                 240                 245
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GGC GTT CTG AAC TAT CCG AT  GTGAGTGATT CCGAAAGTTC CATCGATCAG          1614
Gly Val Leu Asn Tyr Pro Ile
    250                 255

GCTTTCTGAC GCATGAGAAC AG C TAC TAT CCT GCG CTT GAT GCA TTC AAG        1664
                          Tyr Tyr Pro Ala Leu Asp Ala Phe Lys
                                      260

TCT GTC GGC GGC AAT CTC GGC GGC TTG GCT CAG GCC ATC ACC ACC GTG       1712
Ser Val Gly Gly Asn Leu Gly Gly Leu Ala Gln Ala Ile Thr Thr Val
265                 270                 275                 280

CAG GAG AGC TGC AAG GAT TCC AAT CTG CTC GGC AAT TTC CTT GAG AAT       1760
Gln Glu Ser Cys Lys Asp Ser Asn Leu Leu Gly Asn Phe Leu Glu Asn
                285                 290                 295

CAC GAC ATT GCT CGC TTT GCT TC  GTATGGACAC TCTTTTTGAA                 1803
His Asp Ile Ala Arg Phe Ala Ser
            300

GCCCTCATCG ATTGGGGATG CTGACACGGA CAACAACAAC AG G TAC ACG GAT GAC      1858
                                              Tyr Thr Asp Asp
                                              305

CTT GCT CTC GCC AAG AAT GGT CTC GCT TTC ATC ATC CTC TCG GAT GGT       1906
Leu Ala Leu Ala Lys Asn Gly Leu Ala Phe Ile Ile Leu Ser Asp Gly
    310                 315                 320

ATT CCG ATC ATC TAC ACG GGC CAG GAG CAG CAC TAC GCC GGT GAT CAC       1954
Ile Pro Ile Ile Tyr Thr Gly Gln Glu Gln His Tyr Ala Gly Asp His
325                 330                 335                 340

GAT CCC ACA AAT CGT GAG GCC GTC TGG CTG TCT GGC TAC AAT ACC GAC       2002
Asp Pro Thr Asn Arg Glu Ala Val Trp Leu Ser Gly Tyr Asn Thr Asp
                345                 350                 355

GCC GAG CTG TAC CAG TTC ATC AAG AAG GCC AAT GGC ATC CGC AAC TTG       2050
Ala Glu Leu Tyr Gln Phe Ile Lys Lys Ala Asn Gly Ile Arg Asn Leu
            360                 365                 370

GCT ATC AGC CAG AAC CCG GAA TTC ACC TCC TCC AAG GTGAGTACAA           2096
Ala Ile Ser Gln Asn Pro Glu Phe Thr Ser Ser Lys
        375                 380

TAACAAACTT TTCGAAAAAT TTTTCACCGG AGAAAACCTA AGATTCGGCT AACAAAACAA    2156

AAAAAAAAAA G ACC AAG GTC ATC TAC CAA GAC GAT TCG ACC CTT GCC ATT      2206
             Thr Lys Val Ile Tyr Gln Asp Asp Ser Thr Leu Ala Ile
             385                 390                 395

AAC CGG GGC GGC GTC GTT ACT GTC CTG AGC AAT GAA GGC GCC TCC GGG       2254
Asn Arg Gly Gly Val Val Thr Val Leu Ser Asn Glu Gly Ala Ser Gly
        400                 405                 410

GAG ACC GGG ACT GTC TCC ATT CCG GGA ACT GGC TTC GAG GCC GGC ACG       2302
Glu Thr Gly Thr Val Ser Ile Pro Gly Thr Gly Phe Glu Ala Gly Thr
    415                 420                 425
```

```
GAA TTG ACT GAT GTC ATC TCC TGC AAG ACC GTG ACT GCG GGG GAC AGC      2350
Glu Leu Thr Asp Val Ile Ser Cys Lys Thr Val Thr Ala Gly Asp Ser
430                 435                 440                 445

GGG GCG GTC GAC GTG CCC TTG TCG GGC GGA CTG CCA AGC GTG CTC TAT      2398
Gly Ala Val Asp Val Pro Leu Ser Gly Gly Leu Pro Ser Val Leu Tyr
                450                 455                 460

CCC AGC TCC CAG CTG GCC AAG AGT GGT CTG TGT GCG TCG GCG TGA          2443
Pro Ser Ser Gln Leu Ala Lys Ser Gly Leu Cys Ala Ser Ala  *
            465                 470                 475

GGGGATTCTC TCATCACCAG GCGATGTCAG GAGACAATTT TTTTCTGAAC TCAGGGGTTT    2503

CTCAGAGAGC TCAAAATCGG GAACTTTTTT TTTGCAGGAA CTCCGAGTGT ACATATATTG    2563

AGTGGAGTCT CTCATTCGTC TTTTGTGTAG CTTAGGTTGA TCCATAGTGA GTGATTTTTC    2623

ACGTTGCTCC ACGTGGTTTT CTTGACAGTT CGGGCTATGG GGTGTTAGCC TTGTTGGGCA    2683

CGAAGATCCA CACTAAAATC TGACACTATG AAGTCAACTC ACCATCCTGA TCGGATTTGT    2743

GCTCTCATTC CGTCATTTGT CCAATCAACT CTCCGTAAAC CCCCATCCTA ACTCCCTCCC    2803

CAAGTGAACC TATTCCCTTT CTTGTTTCGT AAGCTCCCCA AGGTCCAATG GCCATGTCAC    2863

TTTGATGACG CGTAGCACGT CAGCTGTTCC GTACCTTTTC TCGCCCGATC AGCGGCGGCG    2923

GCGGCGGCGG CGGCGGCAGC AGCAGCAGCA GCAGCAGCAG CAGCAGCAGC AGTCAAGGGG    2983

ACCAAACCAG GAACCCAATT CGGCAGGGAG ACGCGCCGAA TCCGCCGACG TTGAACGCGG    3043

CTAGGAGATT CTTCGCACTT TGTCCCTTTC CCATTGCTCC TCGTCATTGT AGCTTATTCC    3103

TAGTCTCGTT AAACCCGGAA CTAGCGGCCG AATGATTTGG CCACCGCTTC CATACTATGG    3163

TACACCTGCG CTGTACTCCG GACGCAGAAT GTCGGCCTTT ACGCCCCAAT CCCTGTACCT    3223

CGGAGTTATG GTTCGAAGAG TGAATCCTCT TCGATTGCAC ACTTCATGAC ACAGAAGCCG    3283

CTTCGTAATC CCAATCCAAT TGCTGCAG                                       3311
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 494 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Lys Ser Leu Ala Ala Ile Ala Ala Leu Leu Ser Pro Thr Leu Val
-18         -15                 -10                 -5
```

```
Arg Ala Ala Thr Pro Asp Glu Trp Lys Ala Gln Ser Ile Tyr Phe Met
 1               5                  10

Leu Thr Asp Arg Phe Ala Arg Thr Asp Asn Ser Thr Thr Ala Pro Cys
15                  20                  25                  30

Asp Thr Thr Ala Gly Lys Tyr Cys Gly Gly Thr Trp Arg Gly Ile Ile
                35                  40                  45

Asn Asn Leu Asp Tyr Ile Gln Asp Met Gly Phe Thr Ala Ile Trp Ile
            50                  55                  60

Thr Pro Val Thr Ala Gln Trp Asp Asp Asp Val Asp Ala Ala Asp Ala
        65                  70                  75

Thr Ser Tyr His Gly Tyr Trp Gln Lys Asp Leu Tyr Ser Leu Asn Ser
    80                  85                  90

Lys Phe Gly Thr Ala Asp Asp Leu Lys Ala Leu Ala Asp Ala Leu His
95                  100                 105                 110

Ala Arg Gly Met Leu Leu Met Val Asp Val Val Ala Asn His Phe Gly
                115                 120                 125

Tyr Gly Gly Ser His Ser Glu Val Asp Tyr Ser Ile Phe Asn Pro Leu
            130                 135                 140

Asn Ser Gln Asp Tyr Phe His Pro Phe Cys Leu Ile Glu Asp Tyr Asp
        145                 150                 155

Asn Gln Glu Glu Val Glu Gln Cys Trp Leu Ala Asp Thr Pro Thr Thr
    160                 165                 170

Leu Pro Asp Val Asp Thr Thr Asn Pro Gln Val Arg Thr Phe Phe Asn
175                 180                 185                 190

Asp Trp Ile Lys Ser Leu Val Ala Asn Tyr Ser Ile Asp Gly Leu Arg
                195                 200                 205

Val Asp Thr Val Lys His Val Glu Lys Asp Phe Trp Pro Asp Phe Asn
            210                 215                 220

Glu Ala Ala Ala Cys Thr Val Gly Glu Val Phe Asn Gly Asp Pro Ala
        225                 230                 235

Tyr Thr Cys Pro Tyr Gln Glu Val Leu Asp Gly Val Leu Asn Tyr Pro
    240                 245                 250

Ile Tyr Tyr Pro Ala Leu Asp Ala Phe Lys Ser Val Gly Gly Asn Leu
255                 260                 265                 270

Gly Gly Leu Ala Gln Ala Ile Thr Thr Val Gln Glu Ser Cys Lys Asp
                275                 280                 285

Ser Asn Leu Leu Gly Asn Phe Leu Glu Asn His Asp Ile Ala Arg Phe
            290                 295                 300
```

```
Ala Ser Tyr Thr Asp Asp Leu Ala Leu Ala Lys Asn Gly Leu Ala Phe
       305                 310                 315

Ile Ile Leu Ser Asp Gly Ile Pro Ile Ile Tyr Thr Gly Gln Glu Gln
    320                 325                 330

His Tyr Ala Gly Asp His Asp Pro Thr Asn Arg Glu Ala Val Trp Leu
335                 340                 345                 350

Ser Gly Tyr Asn Thr Asp Ala Glu Leu Tyr Gln Phe Ile Lys Lys Ala
                355                 360                 365

Asn Gly Ile Arg Asn Leu Ala Ile Ser Gln Asn Pro Glu Phe Thr Ser
            370                 375                 380

Ser Lys Thr Lys Val Ile Tyr Gln Asp Asp Ser Thr Leu Ala Ile Asn
       385                 390                 395

Arg Gly Gly Val Val Thr Val Leu Ser Asn Glu Gly Ala Ser Gly Glu
    400                 405                 410

Thr Gly Thr Val Ser Ile Pro Gly Thr Gly Phe Glu Ala Gly Thr Glu
415                 420                 425                 430

Leu Thr Asp Val Ile Ser Cys Lys Thr Val Thr Ala Gly Asp Ser Gly
                435                 440                 445

Ala Val Asp Val Pro Leu Ser Gly Gly Leu Pro Ser Val Leu Tyr Pro
            450                 455                 460

Ser Ser Gln Leu Ala Lys Ser Gly Leu Cys Ala Ser Ala  *
       465                 470                 475
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "adaptor upper strand"

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GATCGTTTAA ACGGCGCCG 19

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "adaptor lower strand"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AATTCGGCGC CGTTTAAAC 19

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATGGGNTTYA CNGCNATHTG GAT 23

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTYTGRTANG GRCANGTRTA 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 245 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Thermomyces lanuginosus
(B) STRAIN: CBS 223.63

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:join(2..105, 185..245)

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:106..184

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
T ATG GGG TTT ACG GCG ATA TGG ATT ACT CCA GTG ACA GCC CAG TGG          46
  Met Gly Phe Thr Ala Ile Trp Ile Thr Pro Val Thr Ala Gln Trp
    1               5                  10                  15

GAC GAC GAT GTG GAT GCG GCA GAT GCA ACG TCG TAT CAC GGT TAT TGG        94
Asp Asp Asp Val Asp Ala Ala Asp Ala Thr Ser Tyr His Gly Tyr Trp
                20                  25                  30

CAG AAA GAC CT  GTGCGCAACC CTGCTCCATG GATCGCTGGC TGCAAACTCG          145
Gln Lys Asp Leu
            35

TGCTGATCGG TGATTTTTTT TTTTTTTTTT TTGAAACAG A TAC TCT CTG AAT TCG      200
                                          Tyr Ser Leu Asn Ser
                                                              40

AAA TTC GGC ACT GCC GAT GAC TTG AAA ACC CTG GCT GAT GCC CTT           245
Lys Phe Gly Thr Ala Asp Asp Leu Lys Thr Leu Ala Asp Ala Leu
                45                  50                  55
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Met Gly Phe Thr Ala Ile Trp Ile Thr Pro Val Thr Ala Gln Trp Asp
 1               5                  10                  15

Asp Asp Val Asp Ala Ala Asp Ala Thr Ser Tyr His Gly Tyr Trp Gln
            20                  25                  30

Lys Asp Leu Tyr Ser Leu Asn Ser Lys Phe Gly Thr Ala Asp Asp Leu
        35                  40                  45

Lys Thr Leu Ala Asp Ala Leu
    50                  55
```

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 256 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:join(1..73, 128..256)

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:74..255

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
GTG GAC ACC ACC AAT CCT CAG GTT CGG ACG TTT TTC AAC GAC TGG ATC        48
Val Asp Thr Thr Asn Pro Gln Val Arg Thr Phe Phe Asn Asp Trp Ile
 1               5                  10                  15

AAG AGC CTG GTG GTT AAC TAC TCC A GTATGATTGT TCCCGCGGTA              93
Lys Ser Leu Val Val Asn Tyr Ser
            20

ACGCTTTAGG GCTTGCTCTA ACTGAAATCG ACAG  TC GAT GGT CTG CGC GTC        144
                                     Ile Asp Gly Leu Arg Val
                                      25                  30

GAC ACC GTT AAG CAC GTG GAG AAA GAT TTC TGG CCC GAC TTC AAC GAA      192
Asp Thr Val Lys His Val Glu Lys Asp Phe Trp Pro Asp Phe Asn Glu
                35                  40                  45

GCT GCT GCG TGT ACC GTC GGC GAG GTG TTC AAC GGT GAC CCA GCG TAC      240
Ala Ala Ala Cys Thr Val Gly Glu Val Phe Asn Gly Asp Pro Ala Tyr
            50                  55                  60

ACA TGC CCC TAC CAG A                                               256
Thr Cys Pro Tyr Gln
        65
```

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
Val Asp Thr Thr Asn Pro Gln Val Arg Thr Phe Phe Asn Asp Trp Ile
 1               5                  10                  15

Lys Ser Leu Val Val Asn Tyr Ser Ile Asp Gly Leu Arg Val Asp Thr
            20                  25                  30

Val Lys His Val Glu Lys Asp Phe Trp Pro Asp Phe Asn Glu Ala Ala
        35                  40                  45

Ala Cys Thr Val Gly Glu Val Phe Asn Gly Asp Pro Ala Tyr Thr Cys
    50                  55                  60

Pro Tyr Gln
65
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Ala Thr Pro Asp Glu Trp Lys Ala Gln Ser
1               5                  10
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 17, line s 19–26

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution (*including postal code and country*)

23 St Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
23 June 1994	NCIMB 40655

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (cf Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer R. Mandemaker	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 17, line s 19–26

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution (*including postal code and country*)

23 St Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
23 June 1994	NCIMB 40656

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (cf Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer R. Mandemaker	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 17, line s 19-26

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
23 June 1994	NCIMB 40657

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (cf Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer R. Mandemaker	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 17, line s 19–26

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
23 June 1994	NCIMB 40658

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (cf Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer R. Mandemaker	Authorized officer

CLAIMS:

1. A recombinant enzyme having the following characteristics:

i. α-amylase activity over the range from about 60°C to about 80°C;

ii. a molecular weight of about 54 - 60 kDa when measured by SDS-PAGE, or of about 55265 Da +/- 100 Da when measured by mass spectrometry;

iii. a pI value of about 3.7 (on a Pharmacia IEF gel, 3-9);

iv. a pH optimum of from about 5.8 to about 6;

v. a temperature optimum of from about 60°C to about 70°C.

2. A recombinant enzyme according to claim 1 wherein the enzyme comprises an N-terminal sequence shown as SEQ. I.D. No. 11.

3. A recombinant enzyme according to claim 1 or claim 2 wherein the enzyme comprises the sequence shown as SEQ. I.D. No. 1 or SEQ. I.D. No. 2, or a variant, homologue or fragment thereof.

4. A recombinant enzyme according to any one of the preceding claims wherein the enzyme is expressed by a nucleotide sequence comprising the coding sequence shown in SEQ. I.D. No. 1, or a variant, homologue or fragment thereof.

5. A recombinant enzyme according to any one of the preceding claims wherein the enzyme is expressed by a nucleotide sequence comprising the nucleotide sequence shown as SEQ. I.D. No.1, or a variant, homologue or fragment thereof.

6. A recombinant enzyme according to any one of the preceding claims wherein the enzyme is obtained or obtainable from *T. lanuginosus*.

7. A recombinant enzyme according to any one of the preceding claims wherein the enzyme is expressed by a member of the *Aspergillus* family.

8. A recombinant enzyme according to claim 7 wherein the enzyme is expressed by *Aspergillus niger*.

9. A recombinant enzyme according to any one of the preceding claims wherein the enzyme is obtainable from any one of NCIMB deposit No. 40655, NCIMB deposit No. 40656, NCIMB deposit No. 40657 and NCIMB deposit No. 40658.

10. A recombinant nucleotide sequence comprising the coding nucleotide sequence shown in SEQ. I.D. No. 1, or a variant, homologue or fragment thereof.

11. A recombinant nucleotide sequence comprising the nucleotide sequence shown as SEQ. I.D. No. 1, or a variant, homologue or fragment thereof.

12. A construct comprising the recombinant nucleotide sequence according to claim 10 or claim 11.

13. A vector comprising the recombinant nucleotide sequence according to claim 10 or claim 11 or the construct according to claim 12.

14. A cell, tissue or organism comprising the recombinant nucleotide sequence according to claim 10 or claim 11 or the construct according to claim 11 or the vector according to claim 13.

15. A transgenic organism comprising the recombinant nucleotide sequence according to claim 10 or claim 11 or the construct according to claim 12 or the vector according to claim 13 or the cell, tissue or organism according to claim 14.

16. A transgenic organism according to claim 15 wherein the organism is a filamentous fungus, preferably an *Aspergillus* fungus, preferably *Aspergillus niger*.

17. NCIMB deposit No. 40655, NCIMB deposit No. 40656, NCIMB deposit No. 40657 or NCIMB deposit No. 40658.

18. A method of preparing a foodstuff comprising forming a mixture by contacting starch or a starch based ingredient with an α-amylase and optionally processing the mixture, wherein the α-amylase is a recombinant α-amylase according to any one of claims 1 to 9, or is a recombinant α-amylase expressed by the invention according to any one of claims 10 to 17.

19. A method according to claim 18 wherein the recombinant α-amylase is incorporated into the mixture in an amount sufficient to provide from 50 to 400 units of α-amylase activity per kg of starch or starch based ingredient to form the mixture.

20. A method of preparing a bakery product comprising forming a mixture by contacting a wheat-flour dough with an α-amylase and baking the mixture, wherein the α-amylase is a recombinant α-amylase according to any one of claims 1 to 9, or is a recombinant α-amylase expressed by the invention according to any one of claims 10 to 17.

21. A method according to claim 20 wherein the recombinant α-amylase is incorporated into the dough in an amount sufficient to provide from 50 to 400 units of α-amylase activity per kg of wheat flour used to form the dough.

22. A method according to any one of claims 18 to 21 wherein the foodstuff or bakery product is bread.

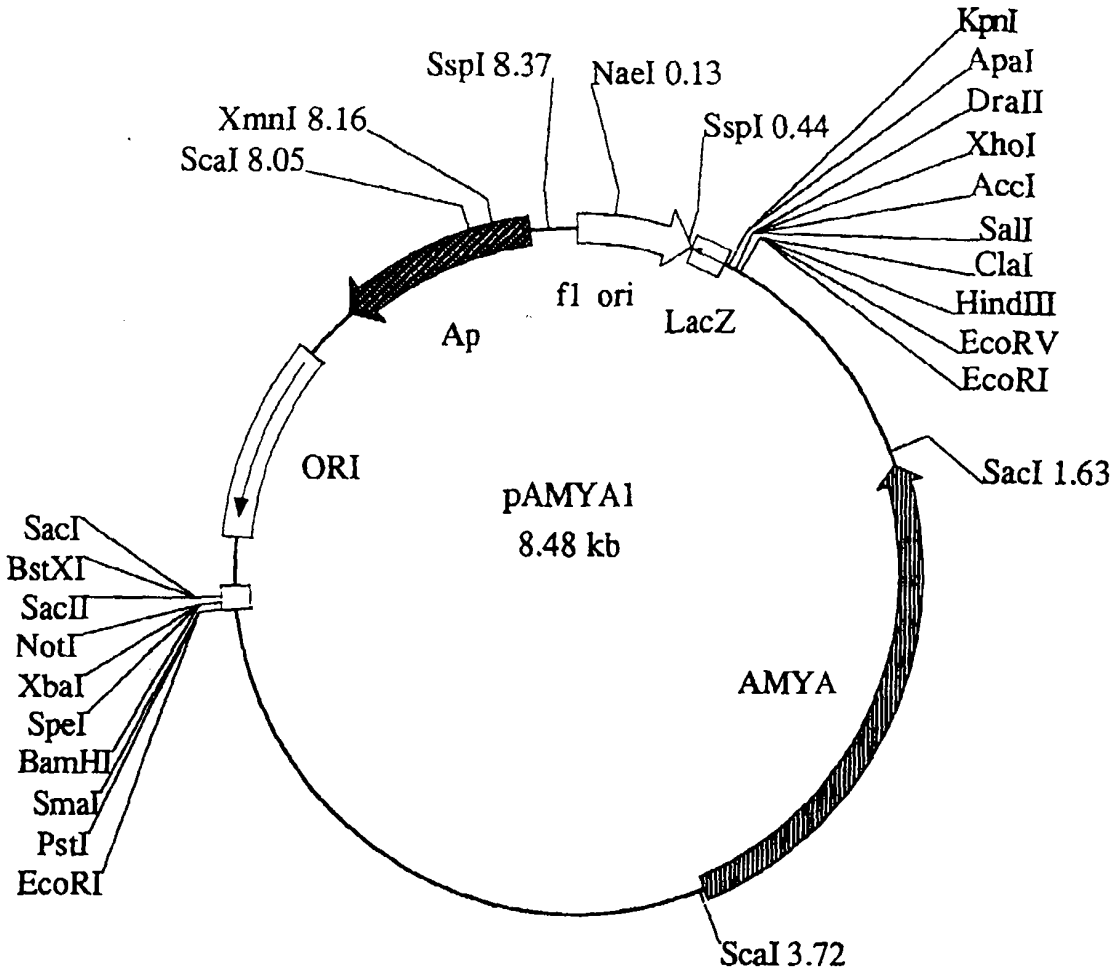
23. A foodstuff obtained by use of the recombinant α-amylase according to any one of claims 1 to 9, or a recombinant α-amylase expressed by the invention according to any one of claims 10 to 17.

24. A bakery product obtained by use of the recombinant α-amylase according to any one of claims 1 to 9, or a recombinant α-amylase expressed by the invention according to any one of claims 10 to 17.

25. Use of a recombinant α-amylase having α-amylase activity over the range from about 60°C to about 80°C in preparing a foodstuff.

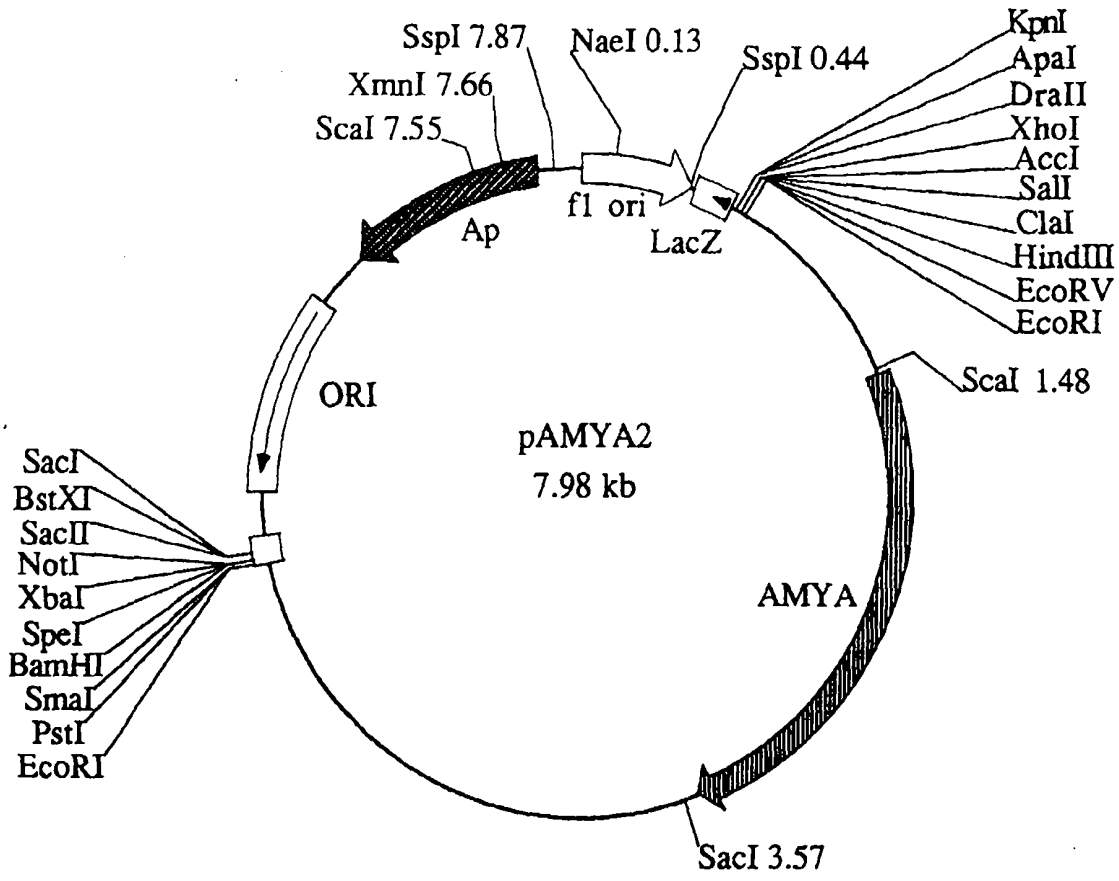
26. Use of a recombinant α-amylase having α-amylase activity over the range from about 60°C to about 80°C in baking.

27. Use according to claim 25 or claim 26 wherein the recombinant α-amylase is a recombinant α-amylase according to any one of claims 1 to 9, or is a recombinant α-amylase expressed by the invention according to any one of claims 10 to 17.



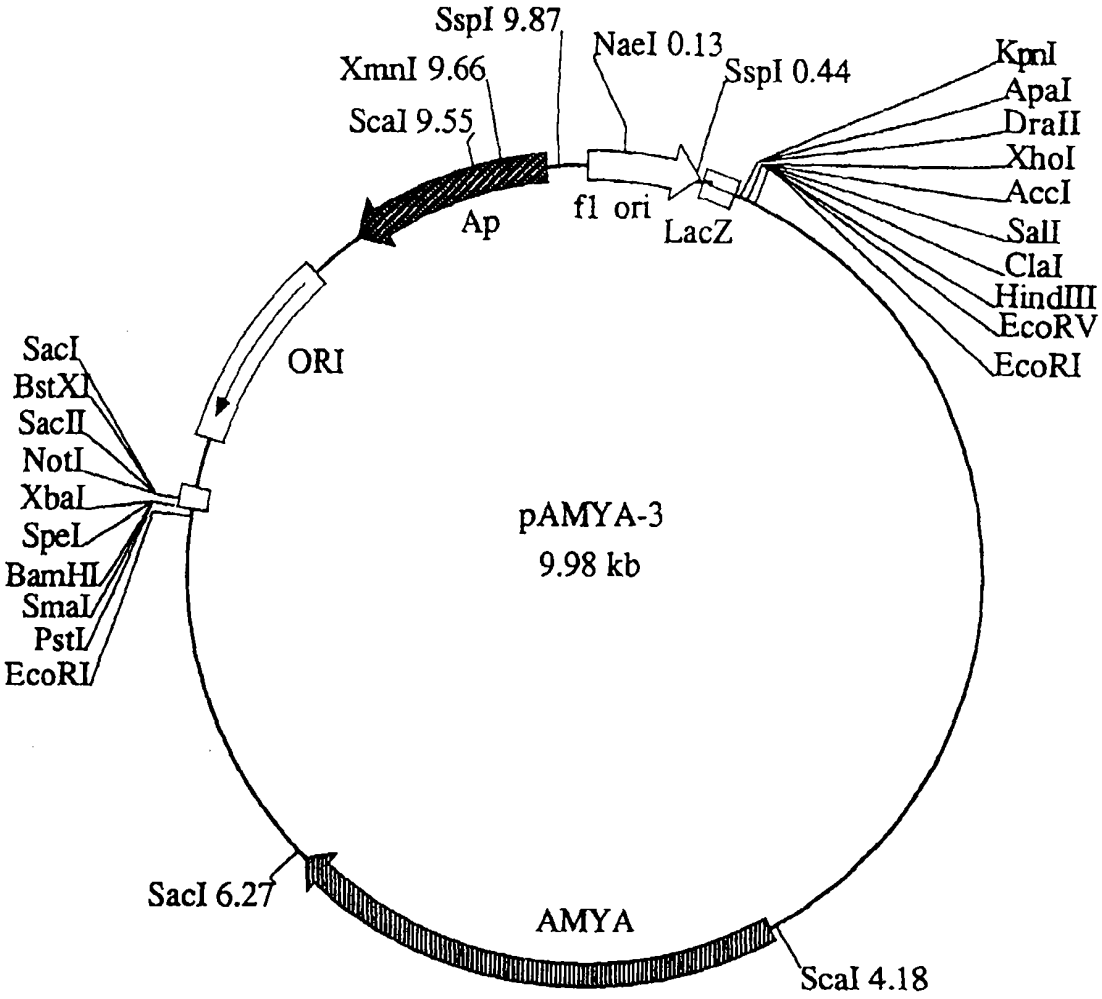
Plasmid name: pAMYA1
Plasmid size: 8.48 kb
Constructed by: PR,JR
Construction date: 15/3 1993
Comments: Made by in vivo excision of an ZAPII phage

Fig 1



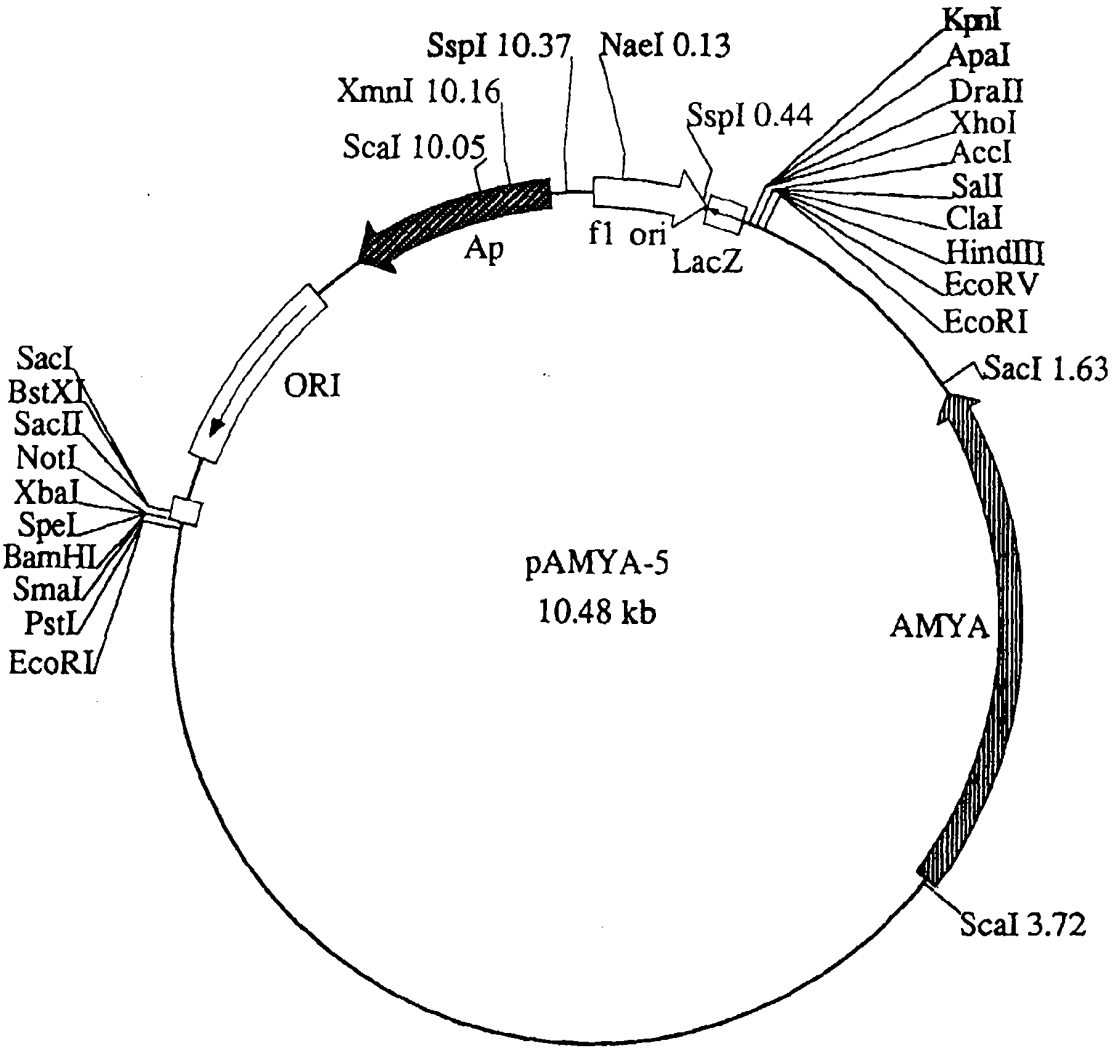
Plasmid name: pAMYA2
Plasmid size: 7.98 kb
Constructed by: PR,JR
Construction date: 15/3 1993
Comments: Made by in vivo excision of an ZAPII phage

Fig 2



Plasmid name: pAMYA-3
Plasmid size: 9.98 kb
Constructed by: PR,JR
Construction date: 15/3 1993
Comments: Made by ion vivo excision of an ZAPII phage

Figure 3



Plasmid name: pAMYA-5
Plasmid size: 10.48 kb
Constructed by: PR,JR
Construction date: 15/3 1993
Comments: Made by in vivo excision of a ZAPII phage

Fig 4

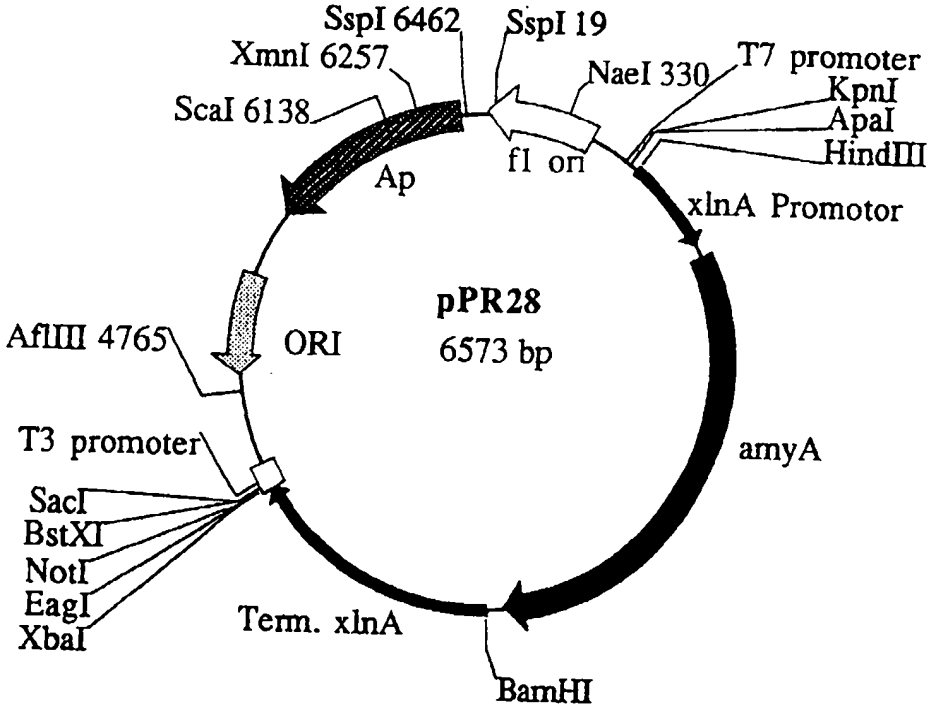

SspI 6462
SspI 19
XmnI 6257
NaeI 330
T7 promoter
KpnI
ScaI 6138
ApaI
HindIII
Ap
f1 ori
xlnA Promotor
pPR28
6573 bp
AflIII 4765
ORI
T3 promoter
amyA
SacI
BstXI
NotI
EagI
XbaI
Term. xlnA
BamHI

Fig 5

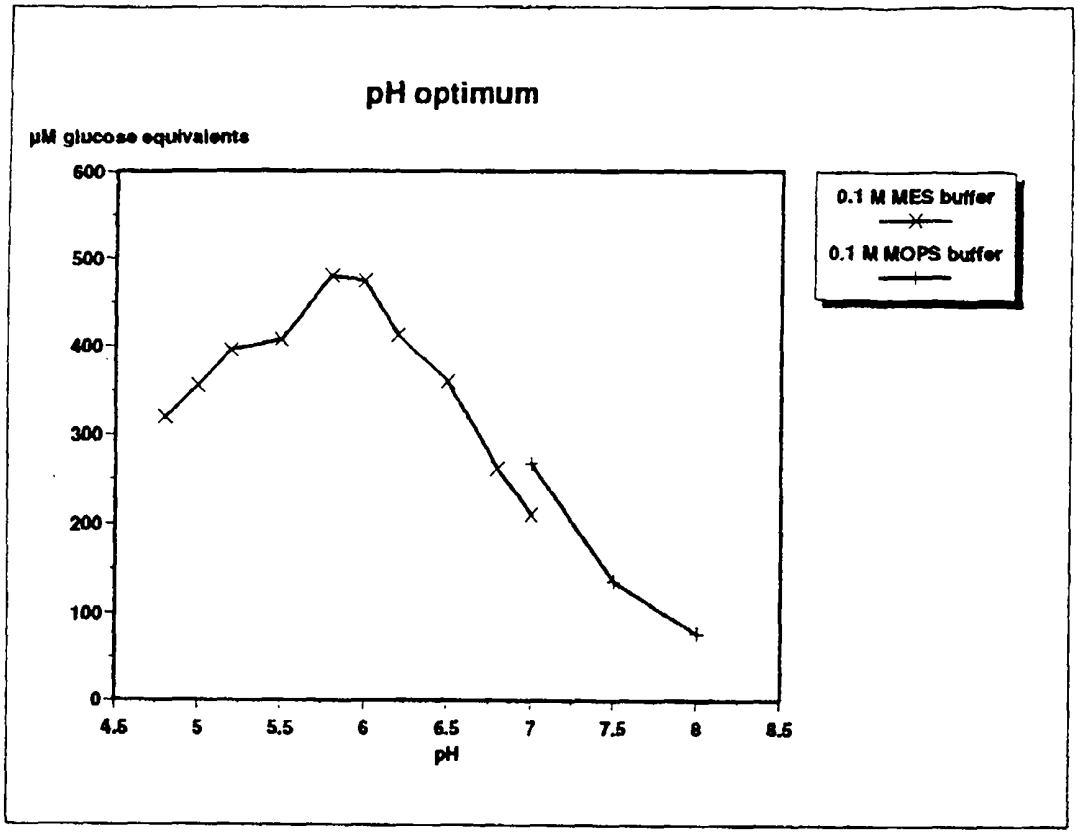

pH optimum
µM glucose equivalents
600
500
400
300
200
100
0
4.5
5
5.5
6
6.5
7
7.5
8
8.5
pH
0.1 M MES buffer
0.1 M MOPS buffer

Fig 6

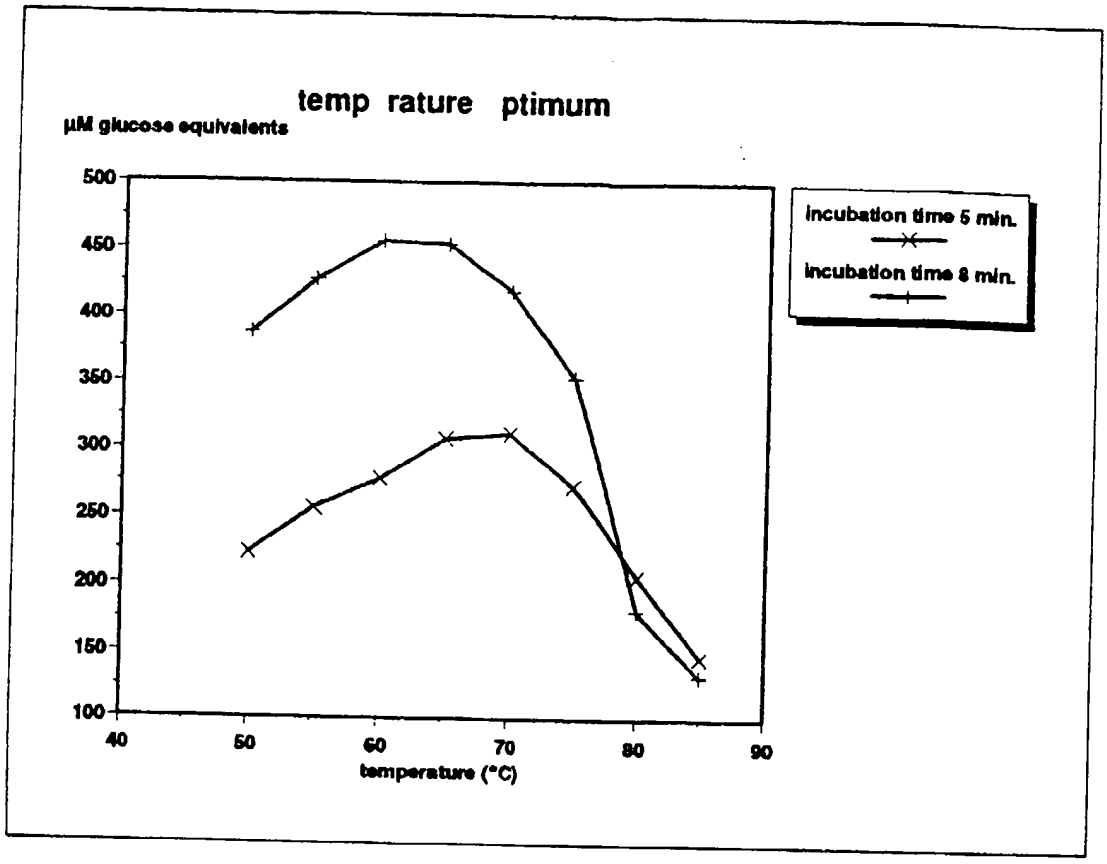

temp rature ptimum
µM glucose equivalents
500
450
400
350
300
250
200
150
100
40
50
60
70
80
90
temperature (°C)
incubation time 5 min.
incubation time 8 min.

Fig 7

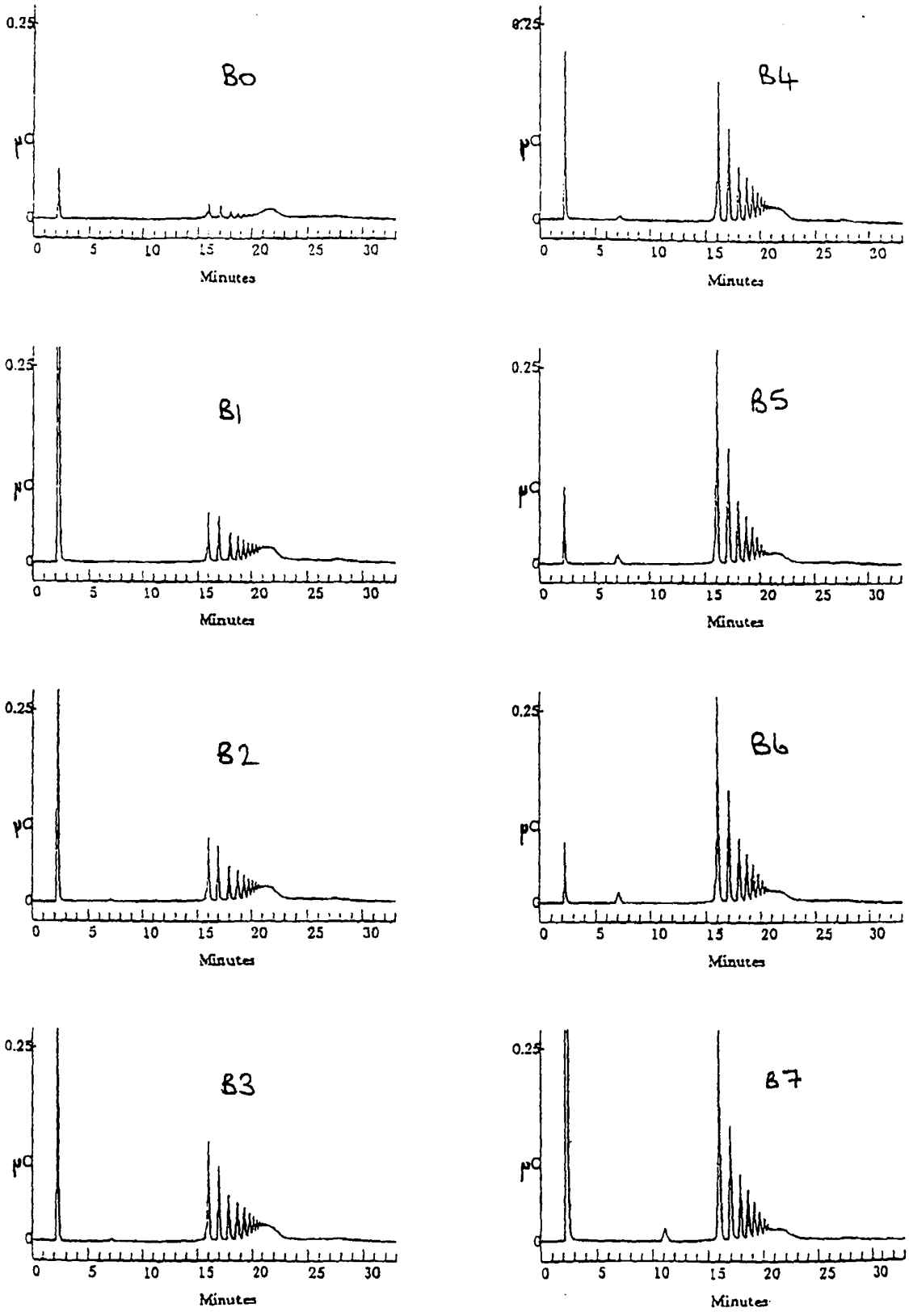


Fig 8

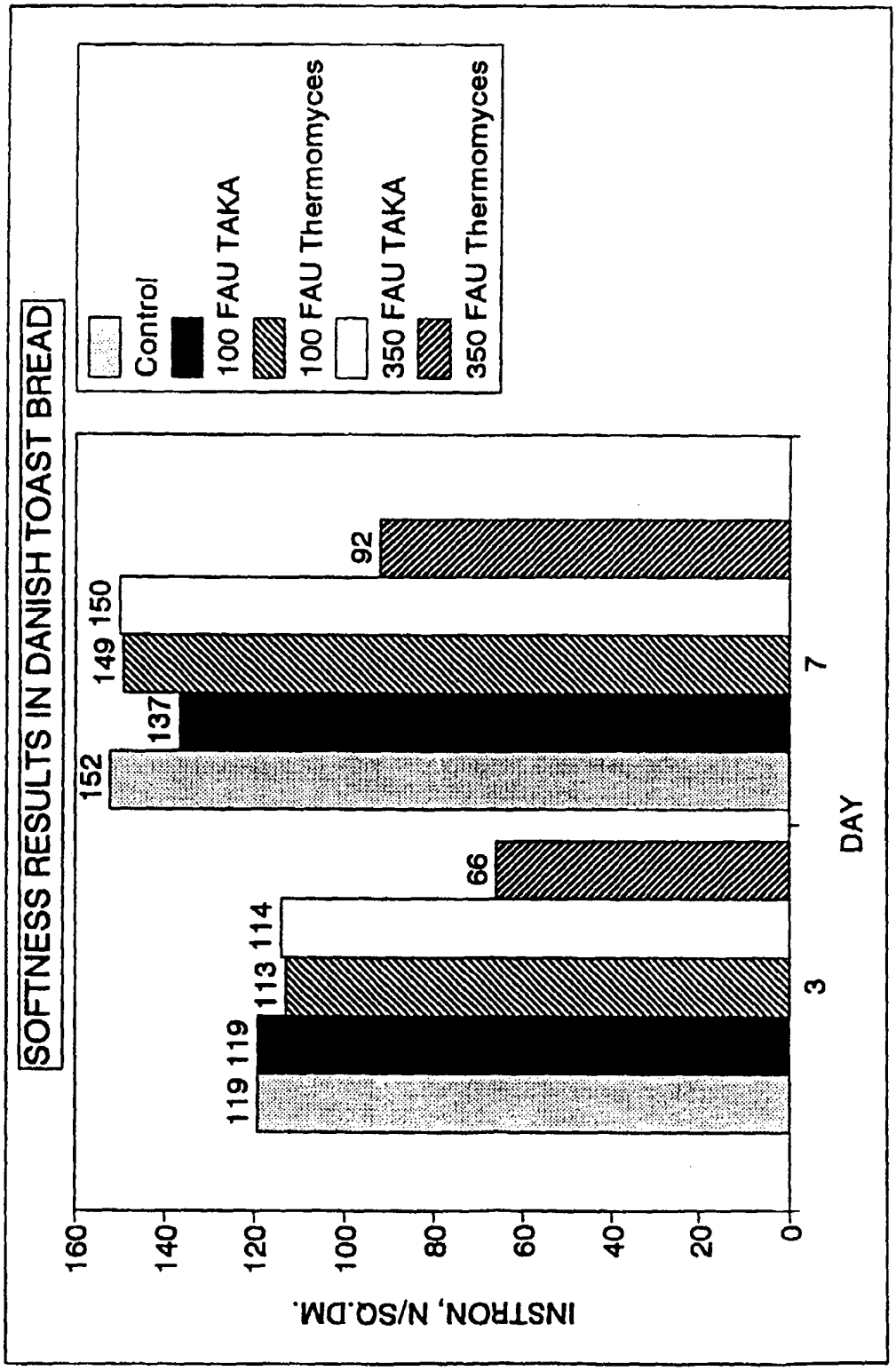

SOFTNESS RESULTS IN DANISH TOAST BREAD
Control
100 FAU TAKA
100 FAU Thermomyces
350 FAU TAKA
350 FAU Thermomyces
INSTRON, N/SQ.DM.
160
140
120
100
80
60
40
20
0
119 119
113 114
66
152
137
149 150
92
3
7
DAY

Fig 9

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/56 C12N1/15 A21D8/04 C12N9/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ENZYME MICROB TECHNOL 14 (2). 1992. 112-116. CODEN: EMTED2 ISSN: 0141-0229, JENSEN B ET AL 'PHYSICOCHEMICAL PROPERTIES OF A PURIFIED ALPHA AMYLASE FROM THE THERMOPHILIC FUNGUS THERMOMYCES -LANUGINOSUS.'	1-17
Y	see the whole document	18-27
Y	US,A,4 416 903 (COLE MORTON S) 22 November 1983 see the whole document	18-27
Y	EP,A,0 273 268 (ENZYME BIO SYSTEMS LTD) 6 July 1988 see the whole document	18-27

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1995	05. 12. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Van der Schaal, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 419 907 (ENZYME BIO SYSTEMS LTD) 3 April 1991 see the whole document ---	18-27
A	EP,A,0 238 023 (NOVO INDUSTRI AS) 23 September 1987 see the whole document -----	1-17

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		JP-A-	63301789	08-12-88
EP-A-0419907	03-04-91	US-A-	5209938	11-05-93
		AU-B-	633138	21-01-93
		AU-B-	6241890	21-03-91
		CA-A-	2025123	14-03-91
		DE-T-	69002572	25-11-93
		ES-T-	2058710	01-11-94
		IE-B-	63138	22-03-95
		JP-A-	3155742	03-07-91
EP-A-0238023	23-09-87	DE-D-	3788524	03-02-94
		DE-T-	3788524	11-05-94
		DK-B-	169134	22-08-94
		EP-A-	0489718	10-06-92
		ES-T-	2061446	16-12-94
		IE-B-	63169	22-03-95
		JP-A-	7051067	28-02-95
		JP-B-	6065316	24-08-94
		JP-A-	62272988	27-11-87